PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : G12Q 1 /02, G01N 33 /53, 33 /50, C07K 16 /00 — A2

(11) International Publication Number: WO 97/22972

(43) International Publication Date: 26 June 1997 (26.06.97)

(21) International Application Number: PCT/US96/19129

(22) International Filing Date: 4 December 1996 (04.12.96)

(30) Priority Data:
60/008,685 15 December 1995 (15.12.95) US

(71) Applicant *(for all designated States except US)*: PHARMACIA & UPJOHN COMPANY [US/US]; 301 Henrietta Street, Kalamazoo, MI 49001 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: TOMICH, Paul, K. [US/US]; 3703 Blackberry Lane, Kalamazoo, MI 49008 (US). JONES, David, A. [US/US]; 1834 Benjamin Avenue, Kalamazoo, MI 49008 (US). FISCHER, Howard, D. [US/US]; 5804 East HJ Avenue, Kalamazoo, MI 49004 (US).

(74) Agent: DARNLEY, James, D., Jr.; Pharmacia & Upjohn Company, Intellectual Property Legal Services, 301 Henrietta Street, Kalamazoo, MI 49001 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: METHOD FOR THE SIMULTANEOUS DETECTION OF A THERAPEUTIC TARGET FOR A DISEASE STATE AND ITS NEUTRALIZING ANTIBODY-LIKE MOLECULE

(57) Abstract

The specification discloses combinatorial libraries of recombinant antibody-like molecules used to identify both potential therapeutic targets and their corresponding neutralizing antibody(ies). The identification can be done either by *in vitro* (biochemical) means or *in situ* with established cell lines. No prior knowledge of either the antigen target or its corresponding antibody is required.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

METHOD FOR THE SIMULTANEOUS DETECTION OF A THERAPEUTIC TARGET FOR A DISEASE STATE AND ITS NEUTRALIZING ANTIBODY-LIKE MOLECULE

FIELD OF THE INVENTION

This invention relates to the field of molecular biology and, more particularly, it relates to a method for the detection of unknown therapeutic targets for a disease state and the simultaneous identification of antibody-like molecules that neutralize these unknown therapeutic targets.

BACKGROUND OF THE INVENTION

Antibody molecules have proven very useful as research tools. They have helped to define protein structure (1,2), been used in a variety of assay formats such as radio-immune assays (3) and enzyme linked immunoassays (4), as modulators of protein-protein interactions (5,6) and recently have been tested for use as therapeutic agents (7,8). Antibodies known to bind to specific antigens, and especially derivatives of smaller mass such as scF_V, have been introduced into eucaryotic cells by a variety of means including micro-injection (9), electroporation (10), and eucaryotic expression vectors (11,12,13). These antibody-like molecules have generated interest for use as potential human gene therapeutic agents (14).

Until recently antibodies have been generated by injection of antigens into animals (15). Currently libraries of various antibody-like molecules have been generated in many laboratories utilizing a variety of methodologies (16-27). Also methodologies have been developed for large scale production of these molecules in tissue cell culture (28) as well as procaryotic cells (23,29). Specific antibodies have been isolated from these libraries which bind to these known, characterized antigens (18,31).

Molecular techniques for inhibiting the expression of specific genes allow a highly refined approach to the analysis and manipulation of microbial and cellular pathways (See review article by Marasco (1995) *Immunotech.* 1:1-19). Intracellular antibodies ('intrabodies') that are synthesized by the cell and targeted to specific cellular compartments represent the most recent innovation in this field, and have been used to inactivate proteins in the endoplasmic reticulum (ER), cytoplasm and nucleus. Endogenous synthesis of the antibodies has allowed a more precise method of intracellular delivery than had earlier methods such as microinjection.

Early molecular studies have demonstrated that coexpression of cytoplasmic heavy and light chains of a neutralizing anti-alcohol dehydrogenase I monoclonal antibody in *Saccharomyces cerevisiae* could provide limited neutralization of enzyme

activity *in vivo*. Similar studies have demonstrated that a catalytic antibody with modest chorismate mutase (EC 5.4.99.5) activity could function inside a yeast cell lacking the natural enzyme, and confer a growth advantage to its host by virtue of its catalytic activity. Secretory, cytoplasmic and nuclear activity of immunoglobulin heavy and light chains has also been demonstrated in mammalian cells.

Recent advances in antibody engineering have allowed antibody genes to be manipulated and antibody molecules to be reshaped. By harvesting the genetic information of the immune system in the form of rearranged immunoglobulin genes, intrabodies of high affinity and fine specificity can be created. These technological advances, combined with the wealth of information that has been obtained on classical intracellular protein trafficking signals, has allowed intrabodies to be directed to and to be functionally active in many different subcellular compartments.

The marriage between these two disciplines has created a new and powerful research tool to analyze and manipulate intracellular proteins. For example, as a research tool, intrabodies can be directed to individual proteins of an infectious agent or cell to identify their important functions *in vivo*. The full realization of the potential of this research tool will be delayed, however, until investigators become more effective at identifying and characterizing *unknown* intracellular proteins which are potential therapeutic targets.

The phage antibody technology is a recently developed technique that allows the expression of monoclonal antibodies on the surface of filamentous phage particles (53,54,55). One advantage of this technology is the linkage of the specificity of a monoclonal antibody with the genetic information encoding the specificity in the same phage particle, thus allowing enrichment of specific phage antibodies by binding to purified antigens attached to a solid phase, followed by elution and amplification of the phage particles (56). Using micropanning or affinity column techniques, researchers have used this technology to generate antibodies reactive to well-characterized antigens that can be purified in large quantities, either as natural or recombinant products, including various haptens, tetanus toxoid, HIV and RSV glycoproteins, HBV core and surface antigens, progesterone, thyroglobulin, TNF alpha, CEA, soluble CD4, IL2R p55 chain, lysozyme, bovine serum albumin, trypsin, anhydrotrypsin, and L6 tumor-associated antigen.

Recently, a method has been reported for identifying, characterizing, and cloning unknown and unpurified cell surface antigens. PCT published patent application WO 94/26787 (27) reports a method using phage antibody technology to generate antibodies directed against previously unknown and unpurified antigens on the surface of intact, whole cells, e.g., as a way to discover new antigens on important cell types.

In the method disclosed by the WO 94/26787 publication, antibodies against previously uncharacterized and unpurified antigens on the surface of target cells in a cell population are generated by incubating a combinatorial library of antibodies expressed on the surface of filamentous phage particles with a target cell population under conditions sufficient to bind a portion of the phage particles to the target cells. The target cells and bound phage particles are then separated from the unbound phage particles, and the bound phage particles are recovered. These phage particles are then amplified to create an enriched library. Monoclonal antibodies specific to the target cell are then isolated from the enriched library for subsequent use.

The WO 94/26787 publication suggests that the cell surface molecules identified using its reported techniques are likely to be mediators of important biological functions and may be used in clinical applications as targets for pharmacological intervention. Unfortunately, this technology can only identify cell surface molecules and must wait to do so until these molecules become present on the cell surface membrane. Further, this technology cannot detect, identify or characterize unknown and unpurified intracellular antigens. Therefore, there remains a need for a method by which unknown and unpurified intracellular antigens can be identified and purified. There further remains a need for a method to simultaneously identify and purify unknown and unpurified surface and intracellular antigens.

SUMMARY OF THE INVENTION

The present invention involves methods for using combinatorial libraries, either created synthetically or by immunization, along with phage antibody technology to identify antibody-like molecules against previously uncharacterized and unpurified antigens from target cells.

In a first embodiment, the method of the present invention comprises the steps of providing a combinatorial library of antibody-like molecules expressed on the surface of filamentous phage particles, the library being generated by immunization of an animal with a homogenate of either diseased or normal target cells. Alternatively, the library is synthesized using combinatorial chemical

techniques. The library is then incubated with a homogenate of normal target cells and, separately, a homogenate of diseased target cells, under conditions sufficient to bind a portion of the phage particles to antigens present in the homogenate of normal target cells to form a first phage bound state and a portion of the phage particles to antigens present in the homogenate of diseased target cells to form a second phage bound state. The first phage bound state is then physically compared to the second phage bound state to identify any unique antigens with their bound phage particles that are present in only one of the first or second phage bound states. The phage particles bound to the unique antigen are then isolated, recovered and amplified to create an enriched library.

In a preferred embodiment, antibody-like molecules specific to the unique antigen can be isolated from the enriched library and sequenced. Incubation of the isolated antibody-like molecule with a homogenate of normal target cells and, separately, a homogenate of diseased target cells, under conditions sufficient to bind the antibody-like molecule to an antigen present in the homogenate of normal target cells or the homogenate of diseased target cells allows the formation of an antibody-like molecule-antigen complex. Following isolation of the complex, the previously uncharacterized and unpurified antigen from target cells can be recovered and sequenced.

In an alternate embodiment, the method of the present invention can confirm a known intracellular antigen as a therapeutic target. In this embodiment, the antibody-like molecules from a scF_V library against a known antigen are expressed as intracellular antibodies in a population of diseased target cells. Target cells that display an altered phenotype are then identified. Where the antigen is unknown, antibody-like molecules from a library generated by immunization of an animal with a culture of diseased target cells are expressed as intracellular antibodies in a population of diseased target cells. Target cells that display an altered phenotype are isolated. The antibody-like molecule from the library responsible for the altered phenotype is identified by e.g., PCR amplification and sequencing.

Further, the present invention provides a synthetic human antibody library of the human antibody *NEWM* (Kabat Antibody Database (Release No. 5, 1991)). This library is obtainable by generating the sequences defined in Table 2 for the heavy chain CDR regions, HCDR2 (residues 50-65) and HCDR3 (residues 98-106), of the heavy chain variable domain for the human antibody *NEWM* amino acid sequence as shown in Chart 2 [SEQ ID NO:3] and for the light chain CDR, LCDR3 (residues 84-92), of the light chain variable domain for the human antibody *NEWM*

amino acid sequence as shown in Chart 3 [SEQ ID NO:4].

BRIEF DESCRIPTION OF THE CHARTS

Chart 1 shows the oligonucleotides [SEQ ID NOS:1 and 2] needed to convert the *Xho*I site of pBPV into an *Sfi*I site compatible with the *Sfi*I site of pCANTAB5E;

Chart 2 shows the amino acid sequence for the heavy chain variable domain for the human antibody *NEWM* [SEQ ID NO:3]; and

Chart 3 shows the amino acid sequence for the light chain variable domain for the human antibody *NEWM* [SEQ ID NO:4].

DETAILED DESCRIPTION

The process of the present invention includes a method to generate antibody-like molecules (e.g., H_V, F_V, scF_V, Fab, $(Fab)_2$ or Ab) against previously unknown and unpurified antigens from target cells. Since these antigens are associated with potential therapeutic targets, binding by an antibody-like molecule to a particular epitope may interfere with those protein(s) being incorporated into the cell membrane or the protein's function in some cellular pathway.

In a first embodiment of the method of the present invention, a combinatorial library of antibody-like molecules expressed on the surface of filamentous phage particles, the library being generated by immunization of an animal with a homogenate of either diseased or normal target cells, or synthesized using combinatorial chemical techniques, is incubated with a homogenate of normal (diseased) target cells under conditions sufficient to bind a portion of the phage particles to antigens present in the homogenate. This interaction with protein(s) from a diseased or normal state can be accomplished using immuno-affinity techniques.

Following this interaction, resolution of the protein(s) from the diseased (normal) state is achieved on a 2-D protein gel. Western blots using residual (soluble) antibody-like molecules are then performed to identify appropriate target(s) (a "protein subtraction" approach). The phage particles bound to these unique antigens are then isolated, recovered and amplified to create an enriched library.

In a second embodiment of the method of the present invention, a combinatorial library of antibody-like molecules is introduced into eucaryotic cell lines which represent diseased and normal states. The antibody-like molecules are expressed from the library as intracellular antibodies (intrabodies) in a population of diseased target cells followed by the screening and/or selection of the transfected cell lines for reversion to normalcy if the cells are in a diseased state and for induction of the diseased state for normal cells (a "screening" approach). Cells that display an

altered phenotype are isolated and the phage particles (or the antibody-like molecule) responsible for altering the phenotype is identified.

The source for the protein antigen(s) for the generation of the antibody-like library can derive from different sources, for example: 1) a protein known to cause a diseased state purified from an appropriate cell line or a diseased animal; or 2) the gene encoding a protein thought to cause a diseased state cloned and expressed in a given host or transcribed/translated *in vitro*, and then purified. The antibody-like recombinant library is expressed in a selected host with appropriate vector(s) for constitutive or regulated expression. Known transcription, translation, splice initiation and termination signals are incorporated as needed. The expressed antibodies in eucaryotic cells reside in the cytosol, however, the expression vectors can be modified with known localization signals (38,39,40,41) to target antibodies to subcellular sites if desired or required.

In addition to generating an antibody library using immunization techniques, combinatorial chemical synthetic methods such as taught in US Patent 5,264,563 to Huse issued 23 November, 1993 are capable of generating antibody libraries with sufficient diversity and affinity to identify these potential therapeutic targets. Briefly, the Huse method involves the synthesis of DNA by triplets of codons followed by subsequent splitting and mixing of resins on which the DNA is synthesized to achieve random, semi-random, or targeted mixtures of oligonucleotides. As shown in Example 3 below, this procedure is used to create a library of oligonucleotides for the heavy chain CDRs, HCDR2 and HCDR3, and the light chain CDR, LCDR3 as defined in the Kabat Antibody Database (36a).

Alternatively, libraries of oligonucleotides for each CDR can be synthesized by trinucleotide-directed mutagenesis (52). In this method trinucleotide phosphoramidites representing codons for all 20 amino acids are prepared as described and then used in automated solid-phase DNA synthesis. A library is generated by adding a mixture of prepared codons representing the panel of amino acids desired at each individual residue position. For example, with reference to Example 3, synthesis of the library of oligonucleotides coding for the LCDR3 region as defined in the Kabat Antibody Database (36a) begins with solid-phase synthesis of the oligonucleotide CAGCAG coding for the two sequential glutamines at residue positions 84 and 85 in LCDR3. Next, the five different trinucleotide phosphoramidites representing the codons for His, Tyr, Trp, Ser and Gly are mixed in equal portions and chemically added to the core CAGCAG oligonucleotide. (Mixtures with different trinucelotide ratios can be used to synthesize

oligonucleotides that code for other, predetermined proportional representation of individual amino acids at given residue positions). For the next position, trinucleotide phosphoramidites representing the codons for Tyr, Thr, Asn and Ser are mixed in equal portions and chemically added to the oligonucleotide. This sequence of steps is continued as required based on the usage of residues at the CDR positions indicated in Table 2.

In either the screening method or the protein subtraction method, any antibodies of interest having insufficient affinity to their respective unknown antigen are optimized by altering the CDRs for both chains using standard molecular biological techniques (e.g., PCR mutagenesis). Further, amplification of recovered phage antibodies yields an enriched phage antibody library. Repeating this enrichment step several times generates a phage antibody library with high specificity for their respective antigens. Each round of enrichment increases the representation of antibodies with higher affinity for the target antigens but decreases the diversity of the library. Thus, the amount of enrichment will depend on the particular requirements of the user.

Once the enriched library is generated, monoclonal antibodies specific for the unknown antigen are isolated from the library. Thus, phage antibodies reactive to the unknown antigen are obtained. The monoclonal antibodies are isolated using methods known and available to those skilled in the art.

Other libraries which can be constructed are: 1) utilization of a different human antibody following the same procedure outlined above; 2) employing the human antibody database for selection of animo acid composition at the residue sites; 3) randomization of different combinations of heavy and light chain CDRs; and 4) some combination of libraries constructed according to 1-3 above.

The cell population used in conjunction with the present method may include whole tissue sections, such as frozen tissue sections, in which the therapeutic target cell population is identifiable. Exemplary methods for identifying and isolating therapeutic target cell subpopulations may include use of immunohistochemical markers, physical location of cell populations, or other methods known and used by those skilled in the art. In practicing the present invention using tissue sections, homogenates of the tissue section are incubated with the phage antibody library under conditions sufficient to bind phage particles to the unknown antigens.

A preferred embodiment of the invention makes use of the phenotypic characteristics of the target cells to identify phage antibodies of interest by means of their binding to antigens from the target cells. In an alternative embodiment, phage

antibodies are selected if they are shown to have an identifiable and preselected function, as compared with other phage antibodies without such function. In either embodiment, phage antibodies may be assayed, for example, for the functional consequences of a phage antibody-antigen binding. One assay may be to measure intracellular calcium concentration. Another effective assay may be used to detect cell activation, cell proliferation, or cell killing, or the blocking of these activities. Yet another assay may be to analyze the expression of preselected genes. Other functional methods of identifying phage antibodies known and available to those skilled in the art may be used.

The antibodies can be used intracellularly or *in vitro* to identify antigens of interest, including integral membrane antigens; utility *in vitro* is obvious *vide supra*; however, with whole cells, the antigen and antibody are selected intracellularly with the antibody recognizing any epitope that would interfere with the antigen being incorporated into the cell membrane (e. g., post-translational modifications, transport, and potentially even the extracellular epitopes themselves) or the protein's function in some cellular pathway.

Proteins in low abundance can be identified using 2-D gels. The gels used in two dimensional gel electrophoresis are blotted onto PVDF membranes (Schleicher and Schull) and probed with pools of an antibody library. Antibody binding was detected with a secondary antibody conjugated to horseradish peroxidase. Protein spots were visualized by enhanced chemiluminescence. Differentially expressed protein spots can be excised. Antibody phage particles can be recovered and then used to infect *E. coli*. Antibody recovered from the infected *E. coli* is used to purify the protein. A cDNA of the protein is obtained by screening a DNA expression library with said antibody.

The invention is further described in the following non-limiting examples. These examples demonstrate first how to use the method of the present invention to identify and characterize "known" therapeutic targets. Later examples then demonstrate how to use the method of the present invention to identify and characterize "unknown" therapeutic targets.

EXAMPLE 1

A. Construction of a "Mouse" scF$_v$ Library

Upon invasion of macrophage, *Shigella flexneri* resides in the cytoplasm (42) and secretes several proteins, one of which is encoded by the *ipaB* gene (43). Since this gene product triggers apoptosis in macrophage (44), it is a potential therapeutic target and thus can be used to demonstrate the method of the present invention.

The first step in generating a library of scF_V antibody molecules is to inject purified *ipaB* into mice. After sacrificing the mice and harvesting their spleens, the poly(A)$^+$ mRNA for murine antibody heavy and light chains is isolated. The heavy and light variable chains are linked together by the Pharmacia Biotech Recombinant Phage Antibody System Mouse scF_V Module (catalog no. 27-9400-01). A library of expressed recombinant anti-*ipaB* scF_V antibodies is generated with the procaryotic expression pCANTAB5E. This plasmid and the means to generate the scF_V library is accomplished with the Pharmacia Biotech Recombinant Phage Antibody System Expression Module (catalog no. 27-9401-01).

Subsequently, they are subcloned into appropriate eucaryotic expression vectors. These vectors, purchased from Pharmacia Biotech but not limited to them, are pBPV (catalog no. 27-4390-01) and pSVK3 (catalog no. 27-4511-01) which have bovine papilloma virus and SV40 origins of replication respectively. The restriction sites for a given eucaryotic expression vector utilized for cloning are converted into appropriate *Sfi*I and/or *Not*I sites compatible with those of pCANTAB5E with designed oligonucleotides to allow subcloning. For example, the *Xho*I site for the pBPV vector is converted to a *Sfi*I site using oligonucleotides 1 and 2 (Chart 1)[SEQ ID NOS:1 and 2]; the *Not*I site remained unchanged. Other vectors would make appropriate changes to allow compatibility of restriction sites for subcloning from pCANTAB5E.

B. Identification of Known Antigen by Screening Approach

The mouse anti-*ipaB* scF_V DNA library subcloned into pBPV is transfected by means of electroporation (50) into the established murine monocyte-macrophage cell line J774A.1, obtainable from American Type Culture Collection (catalog no. ATCC TIB 67). The transfected library is then infected with a virulent strain of *Shigella flexneri*, serotype 2. Virulent strains of *S. flexneri* have been shown to induce apoptosis in macrophages (51). Macrophage cells that survive the apoptotic event are then subcloned and cultured in larger volume.

Total DNAs are obtained from those surviving, transfected cell lines by normal extraction methods (e. g., the Hirt procedure, *Methods in Enzymology* (1971), W. B. Jakoby and I. H. Pastan, eds. 58, pp. 406-408). Using appropriate oligonucleotide primers obtained from Pharmacia Biotech (pCANTAB5E Gene Rescue Primers, catalog no. 27-1581-01), the DNAs which encode the scF_V antibodies are amplified by PCR, isolated and subcloned. In conjunction with Pharmacia Biotech pCANTAB5E Gene Sequencing Primers (catalog no. 27-1585-01), these scF_V DNAs are also sequenced. Similarly, plasmid DNAs containing the insert of interest

can also be isolated directly by PCR from the total DNA preparation. By whichever means, the plasmids containing the scF_V of interest are introduced into macrophage and tested for their ability to block *Shigella flexneri*-induced apoptosis. Those scF_V constructs which most effectively prevent apoptosis are the ones of interest. They are expressed in *E. coli* for use in the purification of the antigen (protein) which would be the causative agent of apoptosis. Since the antibody library is derived with purified *ipaB* antigen, this is the identified protein.

EXAMPLE 2

Identification of Antigens by Protein Subtraction Approach

Depending on the *Escherichia coli* host type and whether co-infection with a wild-type M13 phage is used, the scF_V molecules (*vide supra*, Example 1) are expressed either as soluble antibodies or as fusion molecules of the M13 *gene3* protein. The library of scF_V antibodies are expressed in a suppressor deficient (*sup*$^-$) strain of *E. coli* to generate soluble antibodies in the bacterial periplasmic space. The cells are osmotically shocked and the antibodies partially purified; e. g., a commercial antibody directed against the peptide tag of pCANTAB5E (Biotech Pharmacia catalog no. 27-9412-02) is used for immuno-affinity purification.

Total protein homogenates are prepared from both the J774A.1 monocyte-macrophage cell line and the same cell line infected with *Shigella flexneri*. A homogenate consists of all components of a disrupted cell or any fractions thereof isolated by typical biochemical separation techniques, e.g., extracts of membrane, organelle, nucleus or cytoplasmic fractions. The total protein homogenate for uninfected macrophage cells is mixed with the purified library of soluble scF_V antibodies. Antibody not bound to antigen is separated by size exclusion chromatography using appropriate Sephadex columns. Those antibodies that bind to this protein preparation are collected and discarded. The other fraction containing unbound, soluble antibodies (SA) is retained.

An aliquot of total protein extract obtained from macrophage infected with *Shigella flexneri* is run on 2-D protein gels (45). Western blots are performed using the residual, soluble antibodies (SA). The protein which binds these antibodies is excised from the gel and micro amino acid sequence analysis (46) is used to obtain the protein sequence.

EXAMPLE 3

Construction of a "Human" scF_V Library

The heavy and light chain variable domain sequence of the human antibody *NEWM* (36a) is used as the basis for generating a library of synthetic scF_V

molecules. The amino acid sequence for *NEWM* antibody variable regions is shown in Charts 2 and 3 [SEQ ID NOS:3 and 4]. The heavy chain variable regions include residues 50-65 (CDR2), and 98-106 (CDR3). A *NEWM* scF_V is generated by joining synthetic oligodeoxy-ribonucleotides coding for the heavy and light chain variable region sequences in frame with a synthetic oligonucleotide sequence that codes for a $(Gly_4Ser)_3$ linker peptide. This synthesis of this synthetic DNA oligonucleotide coding for the *NEWM* scF_V protein is the starting point to construct the synthetic scF_V library.

Sequence variation in the library is based on the frequency of usage of different amino acids in a large database for sequences of known murine antibody CDRs at residue positions identified as important for antibody-antigen interactions and/or proper antibody structure (32-36). The amino acid frequency data is compiled from information in published journal articles (32-36) and the Kabat Antibody Database (Release No. 5, 1991)(36a). All available amino acid sequence data for murine antibodies are aligned and quantified to determine the relative frequency for each amino acid at each variable residue position in the initial library. The combination of individual amino acids that represents the minimum number required to represent a plurality of the different species in the database is determined. Table 2 shows the amino acids selected for incorporation at the indicated sites in the CDR regions.

The library is generated with a codon-based, combinatorial chemical synthesis method taught in US Patent 5,264,563 to Huse issued 23 November, 1993. Briefly, the method involves the synthesis of DNA by triplets of codons followed by subsequent splitting and mixing of resins on which the DNA is synthesized to achieve random, semi-random, or targeted mixtures of oligonucleotides. This procedure is used to create a library of oligonucleotides for the heavy chain CDRs, HCDR2 and HCDR3, and the light chain CDR, LCDR3.

For example, synthesis of the library of oligonucleotides coding for the LCDR3 region begins with synthesis on a single batch of resin of two sequential codons for glutamine comprising the nucleotide sequence CAGCAG (for LCDR3 positions 84 and 85). The resin is then split into 5 equal portions representing the 5 different variable amino acids chosen for LCDR3 position 86 as shown in Table 2. The codons used are selected so that none of the restriction sites to be used to clone the library into a desired expression vector are present in the synthetic insert. The actual codons may be different for different vector systems. As one example, the nucleotides C,A,T, coding for His, T,A,T, coding for Tyr, T,G,G, coding for Trp, T,C,T,

coding for Ser, or G,G,T, coding for Gly, are chemically added to the CAGCAG oligonucleotide on the first, second, third, fourth and fifth portions of resin respectively. The five portions are recombined, mixed, and then split again into 4 equal portions representing the variation of the 4 amino acids chosen for position 87 in LCDR3. The nucleotides T,A,T, coding for Tyr, A,C,T coding for Thr, A,A,T, coding for Asn, or T,C,T, coding for Ser are added to the oligonucleotide on the first through fourth portions of resin respectively. The four individual portions of resin are again recombined. The sequence of resin splitting, synthesis of indicated codons, and re-mixing is continued as required based on the usage of residues at the CDR positions indicated in Table 2.

The library of oligonucleotides for each CDR region is used to replace the corresponding CDR region in the parent oligonucleotide coding for the *NEWM* scF_v. The semi-random region in each oligonucleotide mixture is flanked by adjacent sequences from the parent *NEWM* antibody to permit hybridization mutagenesis as described in detail previously (47,48,49). The three CDR regions are replaced sequentially or in combinations. To maximize the diversity in the combinatorial scF_v antibody library, the combinatorial scF_v library contains each of the above three semi-random codon libraries. Appropriate oligonucleotide linkers containing restriction sites such as, but not limited to *Sfi*I and *Not*I are added using standard synthesis or molecular biology techniques for cassette transfer and cloning the library into a suitable vector such as pCANTAB5 (described in Example 1, above). This engineering facilitates the transfer of the entire scF_v library or individual scF_v clones. Appropriate vectors contain, as needed, known transcription and translation initiation and termination signals that allow for constitutive expression in a selected host, although regulated promoters can be employed (37).

EXAMPLE 4

Use of the "Human" scF_v Antibody Library

The procedures described in Example 2 are replicated using the human scF_v antibody library described in Example 3 rather than the mouse scF_v library described in Example 1. In addition to the *ipaB* gene product, the human scF_v library also allows the identification of eucaryotic (macrophage) proteins involved in the apoptotic event downstream of *ipaB*. Antigens identified for the macrophage apoptotic pathway can be compared with the apoptotic pathway in other cell types (See Example 6, *infra*) to allow the identification of a potentially common or specific therapeutic component. This information can prove useful in helping to decide which antigen(s) are potential therapeutic targets.

As in Example 2, the antibodies that bind these antigens are expressed and purified. They are then used to immuno-affinity purify their respective antigens. Subsequently the antigens are sequenced, their genes synthesized, cloned and expressed. A search of protein and DNA databases is performed to see if any identifiable activity might be associated with these antigens, e.g., whether they might be a kinase, protease, polymerase, lipase, glycolase, ligase, etc. With an identified activity, a biochemical assay is developed for use in a drug discovery effort. That is, the purified antigen is tested for small organic molecules that would inhibit activity of the antigen. These small organic molecules are tested to confirm that, like the scF_v antibody, they also inhibit the antigen.

If no homology is ascertained from the databases, biochemical studies are performed to identify a potential function. If no biologic activity is determined for the purified antigen, a possible use of the antigen is to develop a displacement assay. The displacement assay assumes that the antibody, known to block the activity in eucaryotic cells, recognizes an epitope on the antigen essential for its true physiologic activity *in vivo*. In this assay, either the antigen or its cognate scF_v antibody is labeled (e.g., either with a radio-isotopic label (^{125}I) or a fluorescent label (fluorescein isothiocyanate). When these two cognate molecules bind, antigen and antibody, a search for small organic compounds which displaces the labeled material from the unlabeled reagent is performed.

EXAMPLE 5

Identification of Novel Therapeutic Targets

The above examples involve use of a known (pure protein) or unknown target and/or antibodies generated toward these targets. These procedures can be extended for identifying novel therapeutic targets. Identification of proteins critical to p53-induced apoptosis serves as a notable example. The clinical importance of p53-dependent apoptosis is underscored by the presence of dysfunctional p53 in cancers that are refractory to chemotherapy and irradiation (57). In contrast, cancers that respond well to chemotherapeutics and irradiation appear to utilize functional p53 to eliminate targeted cells by induction apoptosis (57). The molecular mechanism for p53-induced apoptosis is unknown.

As discussed in previous examples, transfection of pools of a human scF_v antibody library into cells that undergo p53-dependent apoptosis are used to neutralize and identify critical apoptosis proteins. In this approach, pools containing approximately 10^3 antibody clones are transfected into cultures of MCF-7 breast carcinoma cells. MCF-7 cells (available from the American Type Culture Collection

Bethesda, Maryland under accession number HTB 22) respond to a variety of stimuli by undergoing apoptosis. Apoptosis inducing stimuli include, e.g., tumor necrosis factor, UV radiation and etoposide. Following introduction of antibody pools, the cells are stimulated by exposure to UV radiation and incubated in culture medium to permit death of unprotected cells. Resistant colonies are maintained and expanded by subculturing of distinct colonies. Clonal populations are expanded and potential protective antibodies are identified by PCR amplification and sequencing. this antibody is then used to identify the protein of interest by screening an expression library derived from the original cell model.

EXAMPLE 6

Identification of Interleukin-1 Converting Enzyme as Therapeutic Target

Induction of cell death in many cell types is often accompanied by the activation of a family of proteases with homology to interleukin-1 converting enzyme (ICE). Six human homologues of ICE protease exist to date (58). The role of these proteases in various tissues is uncertain. Following the teachings of the above Examples, an antibody library murine is generated using representative isoforms of this protease family. An *in vitro* protease assay identifies specific inhibitory antibodies directed against the family members (30). Subsequent cloning of these antibodies and transfer to a mammalian expression vector for subsequent expression of these antibodies in various models of cell death. Blockade of cell death with antibodies directed against ICE protease confirms these proteins as potential targets for drug development and demonstrates the specificity of involvement of the various ICE protease family members.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the invention claimed herein.

All cited literature are incorporated by reference in their entirety.

References:

1. Davies, D. R., Padlan, E. A. and Sheriff, S. (1990). Antibody-antigen complexes. *Annual Rev. Biochem.* **59**:439-473.

2. Hibbits, K. A., Gill, D. S. and Wilson. R. C. (1994). Isothermal titration calorimetric study of the association of hen egg lysozyme and the anti-lysozyme antibody HyHEL-5. *Biochem.* **33**:3584-3590.

3. Weinshenker, B. G., Dekaban, G. and Rice, G. P. (1990). Retroviruses and multiple sclerosis. I. Analysis of sero-reactivity by western blot and radioimmune

assay. *Neurology* **40**:1251-3

4. Engvall, E. and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immunochem.* 8:871-879.

5. Tormo, J., Stadler, E., Skern, T., Auer, H., Kanzler, O., Betzel, C., Blaas, D. and Fita, I. (1992). 3-Dimensional structure of the Fab fragment of a neutralising antibody to human rhinovirus serotype-2. *Protein Sci.* **1**:1154-1161.

6. He, X., Ruker, F., Casale, E. and Carter, D. (1992). Structure of a human monoclonal antibody Fab fragment against gp41 of HIV-1. *Proc. Natl. Acad. Sci. USA.* **89**:7154-7158.

7. Van-der-Leij, J. N., Visser, G. H., Bink-Boelkens, M. T., Meilof, J. F. and Kallenberg, C. G. (1994). Successful outcome of pregnancy after treatment of maternal anti-Ro (SSA) antibodies with immunosuppressive therapy and plasmapheresis. *Prenat-Diagn.* **14**:1003-1007.

8. Fagan, E. A.and Singer, M. (1995) Immunotherapy in the management of sepsis. *Postgrad-Med-J.* **71**:71-78.

9. Harrisson, F., Van-Nassauw, L., Van-Hoof, J. and Foidart, J. M. (1993). Microinjection of antifibronectin antibodies in the chicken blastoderm: inhibition of mesoblast cell migration but not of cell ingression at the primitive streak. *Anat-Rec.* **236**:685-696.

10. Lukas, J., Bartek, J. and Strauss, M. (1994). Efficient transfer of antibodies into mammalian cells by electroporation. *J. Immunol. Methods.* **170**:255-259.

11. Marasco, W. A. (1995) Intracellular antibodies (intrabodies) as research reagents and therapeutic molecules for gene therapy. *Immunotech.* **1**:1-19.

12. Biocca, S., Pierandrei-Amaldi, P., Campionj, N. and Cattaneo, A. (1994). Intracellular immunization with cytosolic recombinant antibodies. *Bio/Tech.* **12**:396-399.

13. Werge, T. M., Biocca, S. and Cattaneo, A. (1990). Intracellular immunization: cloning and intracellular expression of a monoclonal antibody to the $p21^{ras}$ protein. *FEBS Letters.* **274**:193-198.

14. Maciejewski, J. P., Weichold, F. F., Young, N. S., Cara, A., Zella, D., Reitz, M. S. Jr. and Gallo, R. C. (1995). Intracellular expression of antibody fragments directed against HIV reverse transcriptase prevents HIV infection *in vitro*. *Nature Med.* **1**:667-673.

15. Harlow, E. and Lane, D. (eds.) Antibodies: A laboratory Manual; Chapter 5, "Immunizations," pp. 53-138. Cold Spring Harbor Laboratory Press (1988).

16. Holliger, P., Prospero, T. and Winter, G. (1993). "Diabodies": Small bivalent and bispecific antibody fragments. *Proc. Natl. Acad. Sci. USA.* **90**:6444-6448.

17. Nissim, A., Hoogenboom, H., Tomlinson, I. M., Flynn, G., Midgley, C., Lane, D. and Winter, G. (1994). Antibody fragments from a 'single pot' phage display library as immunochemical reagents. *EMBO J.* **13**:692-698.

18. Hoogenboom, H., Griffiths, A. D., Johnson, K. S., Chiswell, D. J., Hudson, P. and Winter, G. (1991). Multi-subunit proteins on the surface of filamentous phage: methodologies for displaying antibody (Fab) heavy and light chains. *Nucl. Acids Res.* **15**:4133-4137.

19. WO 92/01047

20. U.S. Patent 4,946,788

21. WO 90/14424

22. U.S. Patent 5,264,563

24. European Patent 440,146

25. WO 92/01787

26. WO 90/14430

27. WO 94/26787

28. U.S. Patent 4,816,397

29. Dueñas, M., Vázquez, J., Ayala, M., Söderlind, E., Ohlin, M., Pérez, L., Borrebaeck, C. A. K. and Gavilondo, J. V. (1994). Intra- and extracellular expression of an scF_V antibody fragment in E. coli: effect of bacterial strains and pathway engineering using *groES/L* chaperonins. *BioTech.* **16**:476-483.

30. Nicholson, D.W. et al. (1995). Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. *Nature* **376**:37-43.

31. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E., Jones, P. T., Low, N. M., Allison, T. J., Prospero, T. D., Hoogenboom, H., Nissim, A., Cox, J. P. L., Harrison, J. L., Zaccolo, M., Gherardi, E. and Winter, G. (1994). Isolation of high affinity human antibodies directly from large synthetic repertoires. *EMBO J.* **13**:3245-3260.

32. Chothia, C., Lesk, A. M., Tramontano, A., Levitt, M., Smith-Gill, S. J., Air, G., Sheriff, S., Padlan, E. A., Davies, D., Tulip, W. R., Colman, P. M., Spinelli, S., Alzari, P. M. and Poljak, R. J. (1989). Conformations of immunoglobulin hypervariable regions. *Nature* **342**:877-883.

33. Chothia, C., Lesk, A. M., Gherardi, E., Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B. and Winter, G. (1992). Structural repertoire of the human V_H Segments. *J. Mol. Biol.* **227**:799-817.

34. Tomlinson, I. M., Walter, G., Marks, J. D., Llewelyn, M. B. and Winter, G. (1992). The repertoire of human germline V_H sequences reveals about fifty groups of V_H segments with different hypervariable loops. *J. Mol. Biol.* **227**:776-798.

35. Walls, P. H. and Sternberg, M. J. (1992). New algorithm to model protein-protein recognition based on surface complementarity: applications to antibody-antigen docking. *J. Mol. Biol.* **228**:277-297.

36. Mian, S., Bradwell, A. R. and Olson, A. J. (1991). Structure, function and properties of antibody binding sites. *J. Mol. Biol.* **217**:133-151.

36a. Kabat, E.A. et al. (1991). Sequences of proteins of immunological interest. 5th Edition, U.S. Department of Health and Human Service, Public Health Service, National Institutes of Health Publication No. 91-3242.

37. Gossen, M., Freundlieb, S., Bender, G. Müller, G. Hillen, W. and Bujard, H. (1995). Transcriptional activation by tetracyclines in mammalian cells. *Science* **268**:1766-1769.

38. Rech, J., Barlat, I., Veyrune, J. L., Vie, A. and Blanchard, J. M. (1994). Nuclear import of serum response factor (SRF) requires a short amino-terminal nuclear localization sequence and is independent of the casein kinase II phosphorylation site. *J. Cell. Sci.* **107**:3029-3036.

39. Munroe, S. and Pelham, H. R. B. (1987). A C-terminal signal prevents secretion of luminal ER proteins. *Cell* **48**:899-907.

40. Duronio, R. J., Rudnick, D. A., Adams, S. P., Towler, D. A., Gordon, J. I. (1991). Analyzing the substrate specificity of Saccharomyces cerevisiae myristoyl-CoA:protein N-myristoyltransferase by co-expressing it with mammalian G protein alpha subunits in *Escherichia coli*. *J. Biol. Chem.* 266:10498-10504.

41. Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J. and Bourne, H. R. (1993). Palmitoylation is required for signaling functions and membrane attachment of Gq alpha and Gs alpha. *J. Biol. Chem.* **268**:25001-25008.

42. Parsot, C. (1994). *Shigella flexnerii*: genetics of entry and intercellular dissemination in epithelial cells. *Curr.Top. Microbiol. Immunol.* **192**: 217-41

43. Menard, R., Sansonetti, P. and Parsot, C. (1994). The secretion of the *Shigella flexneri* Ipa invasins is activated by epithelial cells and controlled by *ipaB* and *ipaD*. *EMBO.J.***13**:5293-5302.

44. Zychlinsky, A., Kenny, B., Menard, R. and Prevost, M. C., Holland, I. B. and Sansonetti, P. J. (1994). *IpaB* mediates macrophage apoptosis induced by *Shigella flexneri*. *Mol. Microbiol.* **11**: 619-627.

45. O'Farrell, P. H. (1975). High-resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.

46. Matsudaira, P. (1987). Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. *J. Biol. Chem.* **262**:10035-10038.

47. Near, RI (1992). Gene conversion of immunoglobulin variable regions in mutagenesis cassettes by replacement PCR mutagenesis. *Biotechniques* 12:88.

48. Glaser, S, Kristensson, K, Chilton, T and Huse, W (1994). Engineering the antibody combining site by codon-based mutagenesis in a filamentous phage display system, In *Antibody Engineering: A practical guide*, CAK Borrebaeck, ed., Oxford University Press, New York, pp. 117-131

49. Yelton, DE, Rosok, MJ, Cruz, G, Cosand, WL, Bajorath, J, Hellstrom, I, Hellstrom, KE, Huse, WD and Glaser, SM (1995). Affinity maturation of the BR96 anti-carcinoma antibody by codon-based mutagenesis. *J. Immunol.* 155(4):1994-2004).

50. McNally, M. A., Lebkowski, J. S., Okarma, T. B. and Lerch, L. B. (1988). Optimizing electroporation parameters for a variety of human hematopoietic cell lines. *Biotechniques* **6**:882.

51. Zychlinsky, A., Prevost, M. C. and Sansonetti, P.J. (1992). *Shigella flexneri* induces apoptosis in infected macrophages. *Nature* **358**:167.

52. Virnekas, B., Ge, L., Pluckthun, A., Schneider, K.C., Wellnhofer, G. and Moroney, S.E. (1994). Trinucleotide phosphoramidites: ideal reagents for the synthesis of mixed oligonucleotides for random mutagenesis. *Nucleic Acids Research* **22**(25):5600-5607.

53. Smith (1985). *Science* **228**:1315-1317.

54. Barbas et al. (1991). *PNAS(USA)* **88**:7978-7992.

55. Clackson et al. (1991). *Nature* **352**:624-628.

56. Kang et al. (1991). *PNAS(USA)* **88**:4363-4366.

57. Lowe, S.W. (1995). Cancer therapy and p53. *Curr. Opin. in Oncology* **7** (in press).

58. Martin, S.J. and Green, D.R. (1995). Protease activation during apoptosis: death by a thousand cuts? *Cell* **82**:349-352.

TABLE 1.

Codon Used	Amino Acid (3 letter code)	Amino Acid (1 letter code)
AAT	Asn	N
CAT	His	H
GAT	Asp	D
TAT	Tyr	Y
ACT	Thr	T
CCT	Pro	P
GCT	Ala	A
TCT	Ser	S
CGT	Arg	R
GGT	Gly	G
TGT	Cys	C
ATT	Ile	I
CTT	Leu	L
GTT	Val	V
TTT	Phe	F
AAG	Lys	K
CAG	Gln	Q
GAG	Glu	E
TGG	Trp	W
ATG	Met	M

TABLE 2. Amino Acids Inserted at Specific Residues in the CDRs for the antibody NEWM.

Region	Residue (amino acids)
LCDR3	84 (Q), 85 (Q), 86 (H,Y,W,S,G), 87 (Y,T,N,S), 88 (E,H,S), 89 (N,L,Y) 90 (P), 91 (R,W,Y,L), 92 (T) 93-103 from antibody NEWM
HCDR2	50 (F,W,N,V,E,R,Y); 51 (I); 52(W,Y,S,D,R,N); 53 (N,P); 54 (Y,N,G); 55 (G,S,N); 56 (G); 57 (G,T,Y,S); 58 (T); 59 (E,Y,N,K); 61(Y), 62 (N), 63 (E), 64 (K), 65,66,67 (VKG & FKG).
HCDR3	95 (E,R,Y,G,S,D); 96 (S,D,R,G,Y); 97 (S,N,D,L,G,Y); 98 (S,N,D,L,G,Y); 99 (D,R,S,Y,G); 100 (F), 101 (D), 102 (Y). 103-117 (taken from known antibody NEWM).

Chart 1

Oligonucleotide 1	5' pTGGCCCAGCGGCCGC 3'
Oligonucleotide 2	5' pGGCCGCTGGGC 3'

Chart 2

```
1    AVQLEQSGPG LVRPSQTLSL TCTVSGSTFS NDYYTWVRQP PGRGLEWIGY

51   VFYHGTSDDT TPLRSRVTML VDTSKNQFSL RLSSVTAADT AVYYCARNLI

101  AGGIDVWGQG SLVTVSS
```

Chart 3

```
1  ASVLTQPPSV SGAPGQRVTI SCTGSSSNIG

31 AGNHVKWYQQ LPGTAPKLLI FHNNARFSVS

61 KSGSSATLAI TGLQAEDEAD YYCQSYDRSL

91 RVFGGGTKLT VLR
```

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Tomich, Paul K.
Jones, David A.
Fischer, H. David

(ii) TITLE OF INVENTION: METHOD FOR THE SIMULTANEOUS DETECTION OF A THERAPEUTIC TARGET FOR A DISEASE STATE AND ITS NEUTRALIZING ANTIBODY-LIKE MOLECULE

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Pharmacia & Upjohn, Inc., Intellectual Property Law
(B) STREET: 301 Henrietta Street
(C) CITY: Kalamazoo
(D) STATE: MI
(E) COUNTRY: USA
(F) ZIP: 49001

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Darnley Jr., James D.
(B) REGISTRATION NUMBER: 33,673
(C) REFERENCE/DOCKET NUMBER: 6002

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 616/385-5210
(B) TELEFAX: 616/385-6897
(C) TELEX: 224401

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 15 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

TGGCCCAGCG GCCGC 15

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGCCGCTGGG C 11

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 117 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Ala Val Gln Leu Glu Gln Ser Gly Pro Gly Leu Val Arg Pro Ser Gln
1 5 10 15

Thr Leu Ser Leu Thr Cys Thr Val Ser Gly Ser Thr Phe Ser Asn Asp
20 25 30

Tyr Tyr Thr Trp Val Arg Gln Pro Pro Gly Arg Gly Leu Glu Trp Ile
35 40 45

Gly Tyr Val Phe Tyr His Gly Thr Ser Asp Asp Thr Thr Pro Leu Arg
50 55 60

Ser Arg Val Thr Met Leu Val Asp Thr Ser Lys Asn Gln Phe Ser Leu
65 70 75 80

Arg Leu Ser Ser Val Thr Ala Ala Asp Thr Ala Val Tyr Tyr Cys Ala
85 90 95

Arg Asn Leu Ile Ala Gly Gly Ile Asp Val Trp Gly Gln Gly Ser Leu
100 105 110

Val Thr Val Ser Ser
115

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 103 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Ala Ser Val Leu Thr Gln Pro Pro Ser Val Ser Gly Ala Pro Gly Gln
1 5 10 15

Arg Val Thr Ile Ser Cys Thr Gly Ser Ser Ser Asn Ile Gly Ala Gly
20 25 30

Asn His Val Lys Trp Tyr Gln Gln Leu Pro Gly Thr Ala Pro Lys Leu
35 40 45

Leu Ile Phe His Asn Asn Ala Arg Phe Ser Val Ser Lys Ser Gly Ser
50 55 60

Ser Ala Thr Leu Ala Ile Thr Gly Leu Gln Ala Glu Asp Glu Ala Asp
65 70 75 80

Tyr Tyr Cys Gln Ser Tyr Asp Arg Ser Leu Arg Val Phe Gly Gly Gly
85 90 95

Thr Lys Leu Thr Val Leu Arg
100

CLAIMS

WE CLAIM:

-1-

A method for identifying antibody-like molecules against previously uncharacterized and unpurified antigens from target cells, the method comprising the steps of:

A. providing a combinatorial library of antibody-like molecules expressed on the surface of filamentous phage particles, said library generated by immunization of an animal with a homogenate of either diseased or normal target cells;

B. incubating said library with a homogenate of normal target cells and, separately, a homogenate of diseased target cells, under conditions sufficient to bind a portion of the phage particles to antigens present in said homogenate of normal target cells to form a first phage bound state and a portion of the phage particles to antigens present in said homogenate of diseased target cells to form a second phage bound state;

C. identifying at least one unique antigen having bound phage particles, said antigen being present in only one of said first or second phage bound states;

D. isolating phage particles bound to said antigen;

E. recovering the phage particles bound to said antigen; and

F. amplifying the bound phage particles to create an enriched library.

-2-

The method of claim 1, wherein Step B comprises:

i. running a two-dimensional gel electrophoresis of said normal target cell homogenate to form a normal material gel and a two-dimensional gel electrophoresis of said diseased target cell homogenate to form a diseased material gel; and

ii. exposing said normal material gel and said diseased material gel to said first library.

-3-

The method of claim 2, wherein Step C comprises:

iii. exposing said normal material gel and said diseased material gel to a secondary labelled antibody; and

iv. identifying differences between said normal material gel and said diseased material gel.

-4-

The method of claim 1, further comprising after Step F the step of:

isolating from said enriched library specific phage particles directed to said antigen.

-5-

A specific phage particle obtained using the method of claim 4.

-6-

A method for identifying a previously uncharacterized and unpurified antigen from target cells, the method comprising the steps of:

A. providing the specific phage particle of claim 5;

B. exposing said phage particle to a heterogenous protein mixture containing said antigen to form a phage particle-antigen complex;

C. isolating said complex; and

D. recovering said antigen.

-7-

An antigen obtained using the method of claim 6.

-8-

The method of claim 1, further comprising after Step F the step of:

isolating from said enriched library antibody-like molecules directed to said antigen.

-9-

A specific antibody-like molecule obtained using the method of claim 8.

-10-

A method for identifying a previously uncharacterized and unpurified antigen from target cells, the method comprising the steps of:

A. providing the antibody-like molecule of claim 9;

B. exposing said antibody-like molecule to a heterogenous protein mixture containing said antigen to form a antibody-like molecule-antigen complex;

C. isolating said complex; and

D. recovering said antigen.

-11-

An antigen obtained using the method of claim 10.

-12-

A method of confirming a known antigen as a therapeutic target in a target cell, the method comprising the steps of:

A. providing a murine scF_v library against a known antigen;

B. expressing at least a portion of said scF_V library as intracellular antibodies in a population of target cells; and

C. identifying target cells that display an altered phenotype.

-13-

A method for identifying antibody-like molecules against previously uncharacterized and unpurified antigens from target cells, the method comprising the steps of:

A. providing a combinatorial library of antibody-like molecules expressed on the surface of filamentous phage particles, said library synthesized by combinatorial chemical means;

B. incubating said library with a homogenate of normal target cells and, separately, a homogenate of diseased target cells, under conditions sufficient to bind a portion of the phage particles to antigens present in said homogenate of normal target cells to form a first phage bound state and a portion of the phage particles to antigens present in said homogenate of diseased target cells to form a second phage bound state;

C. identifying at least one unique antigen having bound phage particles, said antigen being present in only one of the first or second phage bound states;

D. isolating phage particles bound to said antigen;

E. recovering the phage particles bound to said antigen; and

F. amplifying the bound phage particles to create an enriched library.

-14-

The method of claim 13, wherein Step B comprises:

i. running a two-dimensional gel electrophoresis of said normal target cell homogenate to form a normal material gel and a two-dimensional gel electrophoresis of said diseased target cell homogenate to form a diseased material gel; and

ii. exposing said normal material gel and said diseased material gel to said first library.

-15-

The method of claim 14, wherein Step C comprises:

iii. exposing said normal material gel and said diseased material gel to a secondary labelled antibody; and

iv. identifying differences between said normal material gel and said diseased material gel.

-16-

The method of claim 13, further comprising after Step F the steps of:

isolating from said enriched library specific phage particles directed to said antigen.

-17-

A specific phage particle obtained using the method of claim 16.

-18-

A method for identifying a previously uncharacterized and unpurified antigen from target cells, the method comprising the steps of:

A. providing the specific phage particle of claim 17;

B. exposing said phage particle to a heterogenous protein mixture containing said antigen to form a phage particle-antigen complex;

C. isolating said complex; and

D. recovering said antigen.

-19-

An antigen obtained using the method of claim 18.

-20-

The method of claim 13, further comprising after Step F the step of:

isolating from said enriched library antibody-like molecules directed to said antigen.

-21-

A specific antibody-like molecule obtained using the method of claim 20.

-22-

A method for identifying a previously uncharacterized and unpurified antigen from target cells, the method comprising the steps of:

A. providing the antibody-like molecule of claim 21;

B. exposing said antibody-like molecule to a heterogenous protein mixture containing said antigen to form a antibody-like molecule-antigen complex;

C. isolating said complex; and

D. recovering said antigen.

-23-

An antigen obtained using the method of claim 22.

-24-

A synthetic human antibody library of the human antibody *NEWM* (Kabat Antibody Database (Release No. 5, 1991)) obtainable by generating sequences defined in Table 2 for the heavy chain CDR regions, HCDR2 (residues 50-65) and HCDR3 (residues 98-106), of the heavy chain variable domain for the human antibody *NEWM* amino acid sequence as shown in Chart 2 [SEQ ID NO:3] and for the light chain CDR, LCDR3 (residues 84-92), of the light chain variable domain for the human antibody *NEWM* amino acid sequence as shown in Chart 3 [SEQ ID NO:4].

-25-

A method for identifying antibody-like molecules directed against previously uncharacterized and unpurified antigens from target cells, the method comprising the steps of:

A. providing a combinatorial library of antibody-like molecules generated by immunization of an animal with a culture of diseased target cells;

B. expressing the antibody-like molecules from said library as intracellular antibodies in a population of diseased target cells;

C. isolating target cells that display an altered phenotype; and

D. identifying the antibody-like molecule from said library responsible for said altered phenotype.

-26-

The method of claim 25, further comprising the steps of

E. determining the gene sequence of the antibody-like molecule;

F. expressing said gene sequence; and

G. collecting said antibody-like molecule.

-27-

A method for identifying a previously uncharacterized and unpurified antigen from target cells, the method comprising the steps of:

A. providing the antibody-like molecule of claim 26;

B. exposing said antibody-like molecule to a heterogenous protein mixture containing said antigen to form a antibody-like molecule-antigen complex;

C. isolating said complex; and

D. recovering said antigen.

-28-

An antigen obtained using the method of claim 27.

-29-

A method for identifying antibody-like molecules directed against previously uncharacterized and unpurified antigens from target cells, the method comprising the steps of:

A. providing a combinatorial library of antibody-like molecules generated by combinatorial chemical synthesis;

B. expressing said library in a population of normal or diseased target cells;

C. isolating target cells that display an altered phenotype; and

D. identifying the antibody-like molecule from said library responsible for said altered phenotype.

-30-

The method of claim 29, further comprising the steps of

E. determining the gene sequence of the antibody-like molecule;

F. expressing said gene sequence; and

G. collecting said antibody-like molecule.

-31-

A method for identifying a previously uncharacterized and unpurified antigen from target cells, the method comprising the steps of:

A. providing the antibody-like molecule of claim 30;

B. exposing said antibody-like molecule to a heterogenous protein mixture containing said antigen to form a antibody-like molecule-antigen complex;

C. isolating said complex; and

D. recovering said antigen.

-32-

An antigen obtained using the method of claim 31.